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Evaluation of Commercially Available Cold Chain Shipping Systems

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14. ABSTRACT

Air Force Public Health personnel tasked with sampling food suspected of causing illness do not have readily available or standard equipment to perform this task. When presented with a foodborne illness emergency, personnel must scramble to find the necessary sterile tubes, media, and insulated shipping materials. In the present work, three commercially available off-the-shelf cold chain shipping systems were evaluated for temperature holding capacity under the ISCgold™ – Summer and International Safe Transit Association (ISTA)-7D extreme summer temperature shipping profiles. Despite each manufacturer's claims, which indicated that maintenance of temperatures during transport would be highly likely, none of the evaluated boxes were able to sustain sample temperatures between 2°C and 10°C for 96 hours. The NanoCool long haul shipper lasted 55 hours under the ISTA-7D profile and 38 hours under the ISCgold. ThermoSafe's VIP shippers and Pelican BioThermal's Credo Cube performed similarly; VIP shippers maintained sample temperatures under 10°C for 28 hours with the ISTA-7D profile and 15 hours with ISCgold, while the Credo Cube lasted 31 hours under ISTA-7D and 15 hours with ISCgold. Although falling well short of the 96-hour goal, the NanoCool box performed nearly twice as well as the other boxes under the extreme temperature profiles. It has outer dimensions approximately half the size of the other boxes, while providing a similar inner sample space. Additionally, the NanoCool can be "re-iced" during transit lasting longer than 55 hours with a replaceable chilling unit in the lid, making the unit appropriate for the shipment of all types of temperature-sensitive samples.

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1.0 SUMMARY

Currently, Air Force Public Health personnel tasked with sampling food suspected of causing illness do not have readily available and standard equipment to perform this task. When presented with a foodborne illness emergency, personnel must scramble to find the necessary sterile tubes, media, and insulated shipping materials, as well as space on available transportation. The Food Analysis Transport System project will provide Public Health personnel in the U.S. Air Force with a tested shipping container, as well as a detailed operating instruction and protocol for the collection of food samples.

In the present work, three commercially available off-the-shelf cold-chain shipping systems were evaluated for thermal performance under extreme temperature testing profiles created by transportation standards associations. Maintenance of food sample temperatures in a narrow range above freezing (>0°C), but below that of the external environment, is critical for sample integrity and subsequent laboratory testing; the commonly accepted range for perishable samples is 2°C – 10°C. This temperature range prevents freezing and death of microbial pathogens, while inhibiting bacterial growth or degradation of the specimens. In addition to food samples, the tested shipping systems would be appropriate for the regional shipment of a wide variety of temperature-sensitive samples.

None of the evaluated boxes were able to maintain a sample temperature between 2°C and 10°C for the full length of our test. The two passively cooled systems, Thermosafe's VIP shippers and Pelican BioThermal's Credo Cube performed similarly; VIP shippers maintained sample temperatures under 10°C for 28 hours with the 7D profile and 15 hours with ISCgold™, while the CredoCube lasted 31 hours under International Safe Transit Association (ISTA)-7D and 15 hours with ISCgold. The actively chilled NanoCool long haul shipper was the best performing system tested, functioning for 55 hours under the ISTA-7D profile and 38 hours under the ISCgold. Although falling well short of the 96-hour goal, the NanoCool box performed nearly twice as well as the other boxes under the extreme temperature profiles. It has outer dimensions approximately half the size of the other boxes, while providing a similar inner sample space. Additionally, the NanoCool can be "re-iced" during transit lasting longer than 55 hours with a replaceable chilling unit in the lid, making the unit appropriate for the regional shipment of a wide variety of temperature-sensitive samples. While no shipping container met the criteria established for this research project, the NanoCool box provided a suitable, easy-to-use, self-contained, and reusable platform for the shipment of samples based on overnight or 2-day delivery. This may be of benefit for all food sample transport within the continental United States, as well as interregional shipping to local laboratories.

2.0 INTRODUCTION

A recent study by Aronson et al. concluded that several large outbreaks of norovirus and shigella occurred during the initial stages of the second Gulf War. This study and others found that approximately 75% of personnel returning from deployments in Afghanistan or Iraq reported at least one episode of diarrheal disease with up to 50% reporting multiple episodes and 10% reporting persistent episodes that lasted longer than 14 days [1-4]. This impact on the effectiveness of deployed personnel and the drain on effective person-days, combined with recent outbreaks at the Air Force Academy, Kirtland Air Force Base, Hill Air Force Base, and Al Udeid Air Base, illustrates the growing need for more robust food testing capability and

monitoring. Increasingly, difficulties in transporting samples of food suspected to be involved in foodborne illness outbreaks have been documented, including difficulty in securing timely transport, loss of samples in-transit, and arrival of unusable samples at destination due to improper sampling, packaging, shipping, or handling.

The Food Analysis Transport System is intended to fill this gap, providing a compact, insulated, leak-proof, laboratory-approved transport system and operating instruction for sampling and shipping food samples suspected of causing illness, either from unintentional or intentional contamination (bioterrorism event), from remote locations to an appropriate laboratory for analysis. Initially, food samples will be placed in sterile disposable tubes, with a planned minimum capacity of 20 samples per shipment. Future sample preparation will utilize sterile containers with pierceable lids and self-contained blending elements. Blending or homogenizing the sample at the point of collection will permit even, thorough, and rapid cooling of the collected food matrix, as opposed to the temperature gradient and a warmer core commonly observed in food samples.

The appropriate test procedures and parameters for our evaluation of selected commercial off-the-shelf shipping systems in this work were taken from the industry-recognized International Safe Transit Association (ISTA). This organization focuses on transport packaging standards, test protocols, and testing laboratory certification. It develops and publishes test procedures and standards for package integrity and strength, as well as thermal performance under normal and extreme environmental conditions. The ISTA-7D test procedure was chosen as one of the two shipping profiles to evaluate the temperature holding capacity of the selected commercial off-the-shelf shippers. The other high temperature profile, ISCgold™ – Summer (Tegrant Corp., ThermoSafe Brands, Arlington Heights, IL), was developed by an ISTA-certified laboratory as a more extreme alternative to ISTA-7D. While both profiles have mean temperatures >30°C for the duration of the test, ISCgold – Summer is a more severe test of a shipper's thermal performance [5]. Each summer temperature profile was programmed into a Binder KBF constant climate chamber to ensure consistent time and temperature changes as dictated by the different summer thermal profiles. A detailed description of each shipping profile can be found in section 3.2.2 of this report. Packed boxes were subjected to the extreme summer temperature profiles multiple times; internal temperatures of the boxes and samples were tracked with American Thermal Instruments temperature loggers and the organisms assayed for differences in viability.

3.0 MATERIALS AND METHODS

3.1 Culture/Stock Strain Preparation

Surrogate bacterial strains for Gram-negative pathogens (*Escherichia coli*; American Type Culture Collection (ATCC) #BAA1427) and Gram-positive pathogens (*Bacillus atrophaeus Nakamura*; ATCC#9372) were grown to confluence in Terrific broth (TB) with shaking at 37°C overnight from well isolated colonies streaked from reference stocks. Stock aliquots were prepared by adding equal volumes of a filter-sterilized 50% glycerol solution and frozen at -80°C. Liquid culture stocks were thawed on ice after 24 hours and serially diluted from 10^{-1} to 10^{-11} for enumeration on TB agar plates and incubated overnight at 37°C. Plates with 30-300 colonies were counted and the concentration of the stock culture in colony forming units (CFU) per mL was calculated as follows: CFU/mL = (# colonies)*(dilution factor) /

(volume plated in mL). MS2 bacteriophage (ATCC #15597-B1) stocks were prepared and enumerated as recommended by the ATCC package insert. Briefly, MS2 is prepared and viability is assayed similarly to bacterial samples on TB agar, but with a top layer of low percentage agar (0.5%) inoculated with the host strain of the phage (*Escherichia coli* C-3000; ATCC #15597) to create a confluent lawn against which lysed areas (plaques) can be counted. Stocks were diluted to a final concentration of 10^6 cells/mL the day of each experiment.

3.2 Shipping Systems, Ambient Temperature Profiles, and Packing

3.2.1 Shipping Systems. Three insulated shipping systems were chosen from commercially available and industry tested candidates that met ISTA criteria for shock, vibration, and compression. Thermosafe VIP shippers and Pelican Biothermal Credo Cube utilized passive cooling systems, i.e., phase-change gel-packs or panels preconditioned to 4°C. The final system, NanoCool long haul shippers, used an active evaporative cooling system contained in disposable/replaceable lids to maintain a refrigerated temperature range. Passive cooling components (gel-packs, panels, and ballast) were preconditioned to 4°C for ≥24 hours; all systems were utilized as per the manufacturer's instructions.

3.2.2 Ambient Temperature Profiles. Ambient temperature profiles for the 96-hour test were taken from standard industry profiles for the validation of shipping containers during the summer months: ISTA's 7D summer profile and ThermoSafe's ISCGold – Summer profile [5,6]. Both profiles are considered "extreme" ambient temperature profiles with average temperatures of 30.4°C for the 7D profile and 31.8°C for the ISCgold profile. Profiles were programmed into a Binder KBF720 constant climate chamber in repeating cycles for 48 or 96 hours without humidity control and subjected to each temperature profile at least three times (Table 1) [7].

Table 1. Ambient Temperature Profiles

Profile	Profile Step	Temperature (°C)	Time (h:min:s)
ISTA-7D	1	22	04:00:00
	2	35	02:00:00
	3	30	36:00:00
	4	35	06:00:00
ISCgold – Summer	1	38	01:00:00
	2	30	11:00:00

Note: ISTA-7D is a 48-hour protocol and is run twice for the 96-hour trial, while the ISCgold is a 12-hour protocol and is repeated eight times.

3.2.3 Packing and Temperature Logging. One-mL aliquots of each organism were packed in triplicate, in the center of each shipping system between layers of ballast (i.e., preconditioned gel-packs or 50-mL tubes of water) and adjacent to the temperature probe (American Thermal Instruments Logic X2 trackers). Aliquots of each organism were placed in a refrigerator at 4°C to serve as the baseline control for enumeration and viability at the start of each experiment.

3.3 Sample Treatment

3.3.1 Post-Incubation. Immediately following the 96-hour incubation in the environmental chamber, boxes were unpacked and samples placed at 4°C to arrest potential outgrowth of bacterial samples. Temperature probes were read prior to processing to ensure that samples within the boxes remained within temperature (2°C – 10°C). One-mL samples were split into two 500-µL aliquots; one aliquot of the bacterial samples was serially diluted and plated on TB agar for a viable cell count as in stock preparation and the other was processed to extract nucleic acids for quantitative real-time polymerase chain reaction (qRT-PCR). Dilution series were incubated overnight at 35°C; plates with 30 – 300 colonies or plaques were counted and recorded. Viability was compared to control samples that remained at 4°C. DNA and RNA were extracted utilizing either the Life Technologies ChargeSwitch® total RNA (MS2) or Dynabeads® DNA Direct™ Universal (*B. atrophaeus* and *E. coli*) kit according to the manufacturer's instructions.

3.3.2 PCR Conditions. Extracted nucleic acids were amplified on Jena Analytik TProfessional thermal cyclers under the following protocol with either Qiagen OneStep reverse transcriptase PCR or HotStarTaq *Plus* DNA Polymerase as appropriate and according to kit instructions:

1. 48°C – 30 min – Reps 1 (reverse transcriptase step)
2. 95°C – 15 min – Reps 1 (denaturation/hot-start step)
3. 95°C – 30 s – Reps 40 (amplification step)
   50°C – 30 s
   72°C – 1 min
4. 72°C – 10 min (final extension)

The PCR primers listed below (Table 2) were used in both amplification PCR and qRT-PCR.

Table 2. PCR Primers

Sequence (5' – 3')	Primer Name
CCAACAGTCTGGGTTGCCAC	g1-MS2-1534R
CGTTCACAGGCTTACAAAGTAACCT	g1-MS2-1418F
CGTATTCACCGCGGCATG	sRb-BacSp1350R
TCACCAAGGCRACGATGCG	sRb-BacSp0255F
CAGTACAGGTAGACTTCTG	tuf-Ec0754R
TGGGAAGCGAAAATCCTG	tuf-Ec0553F

3.3.3 Real-Time PCR Conditions. Quantitative real-time and reverse transcriptase PCR (qRT-PCR and RT-PCR) was performed on an Applied Biosystems 7500 Fast Dx instrument, utilizing Applied Biosystems' Power SYBR Green (DNA) or Power SYBR Green RNA to CT (RNA) kits according to manufacturer's instructions. PCR cycle conditions are below:

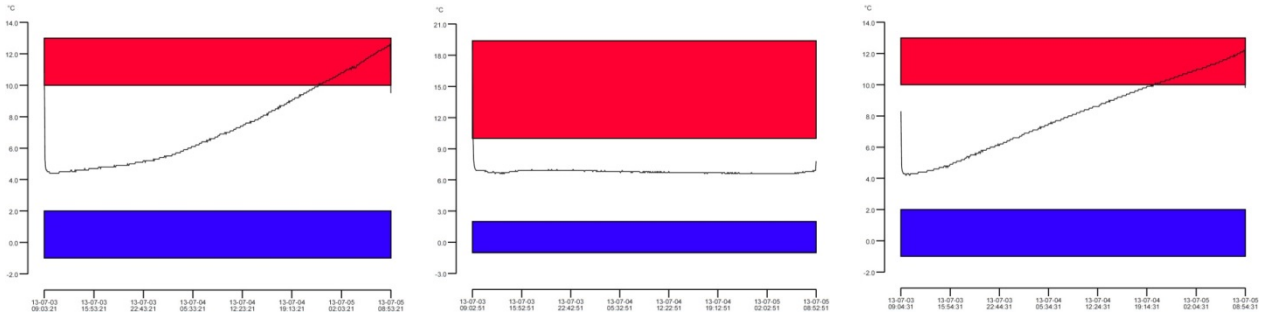
1. 48°C – 30 min – Reps 1 (reverse transcriptase step)
2. 95°C – 10 min – Reps 1 (denaturation/hot-start step)
3. 95°C – 15 s – Reps 40 (amplification step)
   50°C – 1 min
4. 95°C – 15 s – Reps 1 (disassociation/melt curve step)
5. 60°C – 1 min
6. 95°C – 15 s

3.3.4 Homogenization. The Ultra-Turrax tube-drive homogenizer from IKA, which includes a powered base and disposable tubes containing a rotor-stator element, was utilized according to manufacturer's instructions for the maceration of heart tissue. Briefly, 10 g of ground beef was placed alone or with 40 mL 4°C phosphate buffered saline (PBS) in a 50-mL IKA Turrax tube and the temperature of the samples was tracked with and without homogenization in a 4°C refrigerator. Turrax homogenized samples were compared to samples hand stomached for 60 seconds as a control. Temperature was tracked with a standard bi-metallic probe and/or an American Thermal instruments logger with extended probe.

4.0 RESULTS

4.1 Temperature Holding Capacity

Industry certified and commercially available insulated shipping systems were evaluated for their ability to maintain a sample temperature of between 2°C and 10°C for 48 and 96 hours under "extreme" summer temperature profiles. NanoCool (NanoCool LLC, Albuquerque, NM), Credo Cube (Pelican BioThermal, Plymouth, MN), and VIP shippers (Sonoco ThermoSafe, Arlington Heights, IL) systems were incubated under the ISTA-7D and Sonoco ThermoSafe's ISCgold – Summer profiles with near-field capable (NFC) temperature loggers packed adjacent to samples. Of the tested boxes, only the NanoCool system was capable of maintaining a sample temperature between 2°C and 10°C for more than 38 hours (Figure 1) under either summer profile. None of the tested boxes were capable of maintaining temperature for the full 96-hour incubation, under either summer shipping profile (Figure 2). The NanoCool container maintained sample temperature under 10°C for 38 and 55 hours on the ISCgold and ISTA-7D profiles, respectively. The Credo Cube and ThermoSafe products performed similarly, with both containers failing after approximately 15 hours under the ISCgold profile and 30 hours under the ISTA-7D. To prolong the temperature holding capacity of the tested boxes to reach the minimum 4 days, mitigation efforts were implemented on the best performing insulated container, the NanoCool shipping system. NanoCool boxes were incubated under the severe temperature profiles wrapped in foil or placed inside an additional uninsulated box.



(A) Credo Cube (B) NanoCool (C) ThermoSafe

Figure 1. Temperature curves of the candidate shipping systems incubated for 48 hours under the ISTA-7D summer profile. Areas in red are above the maximum holding temperature of 10°C, areas in blue below 2°C.

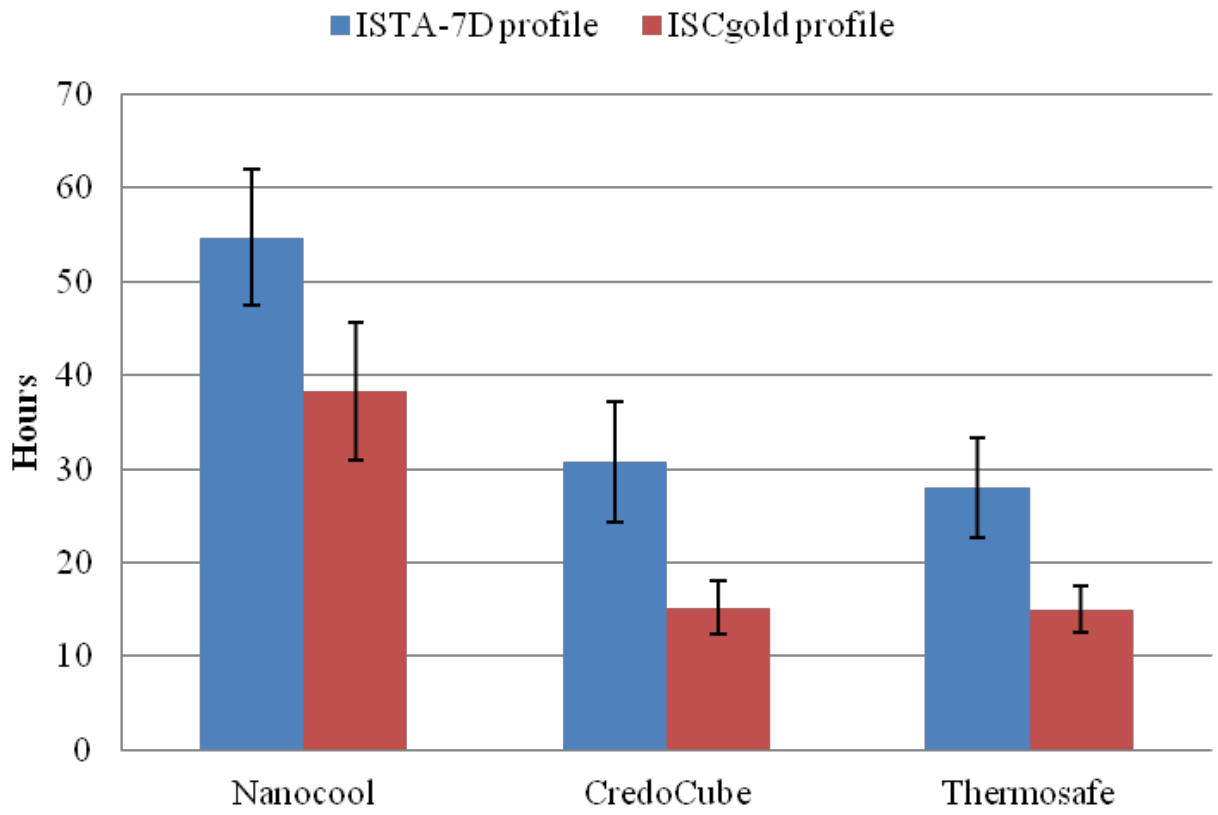
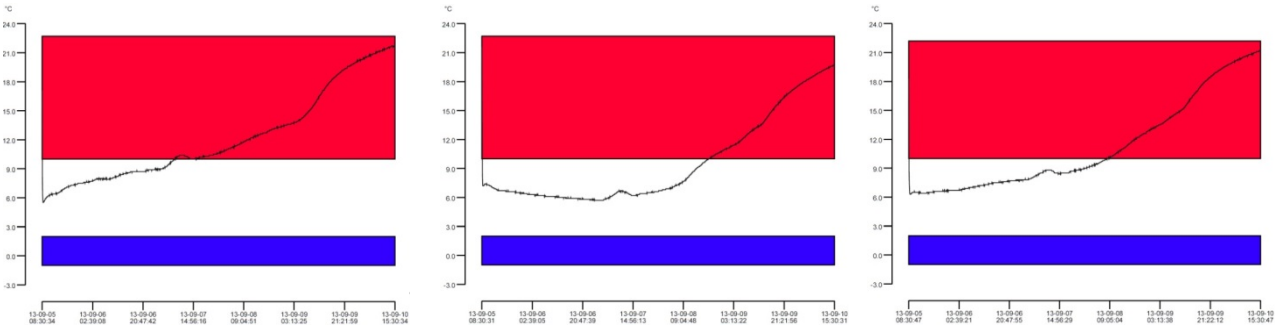


Figure 2. Shipping container performance as measured by the time required for sample chambers to warm to 10°C. Blue bars are boxes incubated under the ISTA-7D summer shipping profile, red bars the ISCgold – Summer profile.

4.2 NanoCool Mitigation Efforts

As the best performing insulated box, simple modifications in addition to manufacturer's instructions were tested on the NanoCool container under the ISTA-7D profile to explore if improvements in the temperature holding capacity of the container were possible. Boxes were wrapped in foil or placed inside an additional cardboard box with foam spacers to create additional insulating capacity. Mitigation efforts were effective in stretching the temperature holding capacity of the NanoCool box, resulting in an increase to 79 hours with the double-boxed container and 88 hours with the foil-wrapped box, as compared to the unmodified control box (Figure 3). However, even with modification, the NanoCool box was unable to maintain the correct sample temperature for the full 96-hour incubation under the ISTA-7D profile. Under mild temperatures (room temperature ~25°C) the container maintained a sample temperature of less than 10°C for 102 hours, surpassing the 96-hour goal, albeit at much lower temperatures than the extreme weather profiles (data not shown).



(A) NanoCool Control (B) NanoCool – foil wrapped (C) NanoCool – double boxed

Figure 3. Temperature curves of the NanoCool insulated container incubated for 96 hours under the ISTA-7D summer profile. Areas in red are above the maximum holding temperature of 10°C, areas in blue below 2°C.

4.3 Quantitation of Microbe Survival/Growth

With the exception of the NanoCool box under the 48-hour ISTA-7D incubation, none of the boxes tested were able to maintain a sample temperature under 10°C for the complete duration of the incubation. Samples from an early failed run were plated on solid media and analyzed by PCR for differences in microbe growth and viability between the tested shipping systems. However, all bacterial samples were overgrown and unable to be enumerated, as measured by both plate growth and qRT-PCR (data not shown). Viable MS2 bacteriophage levels were unchanged by the incubation, as the bacterial virus was not expected to replicate or experience significant decreases of viability during the course of the experiments. Samples from subsequent failed experiments (i.e., recorded sample temperatures >10°C) were not analyzed by PCR as the expected outgrowth of bacterial cells obscured differences by plate count and qRT-PCR.

4.4 Homogenization

Ten grams of room temperature (25°C) raw ground beef was placed in a 50-mL IKA Turrax tube with rotor-stator elements and 40 mL of 4°C PBS. Temperature probes were placed in the center of the ground beef to record the starting temperature and removed during the homogenization process. Turrax homogenization was compared to hand- and machine-stomached samples as controls. The center of unhomogenized samples was still significantly above 10°C after 15 minutes in the pre-chilled PBS and a 4°C refrigerator. In contrast, Turrax homogenized samples were chilled to approximately 10°C by the end of the 59-second maceration. Samples stomached by hand took approximately 10 minutes to chill to 10°C and were much more heterogeneous than the IKA samples. However, difficulties in finding an accurate thermometer appropriate for this task made quantization of the temperature differences inaccurate and the results qualitative.

5.0 DISCUSSION

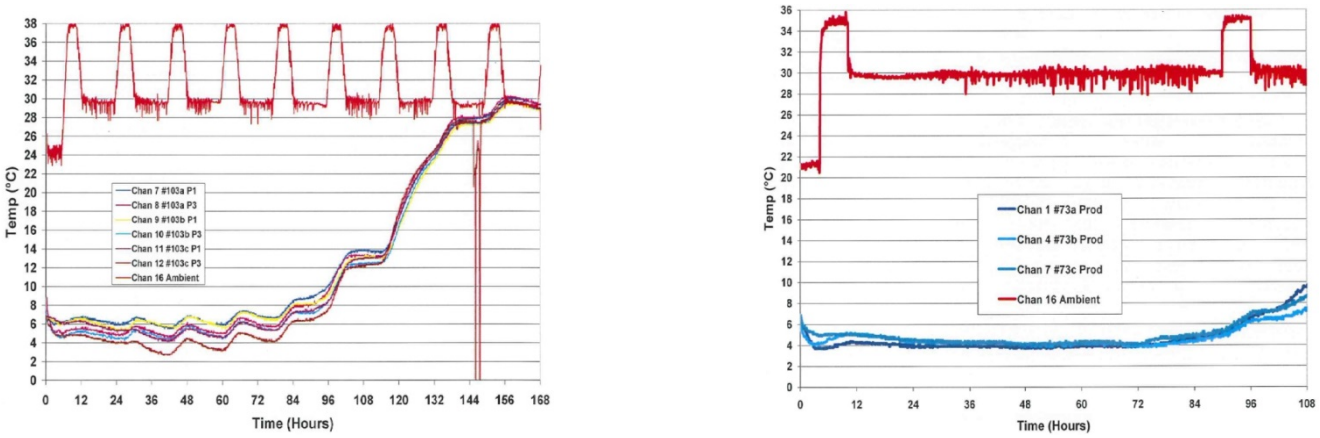
5.1 Failure to Maintain Sample Temperature

Maintenance of sample temperature while being shipped is a critical concern in a wide variety of circumstances and events. Food samples suspected of causing illness must remain under 10°C until receipt and analysis to prevent growth of bacterial organisms and degradation of viral pathogens. Three commercially available cold chain containers, advertised and sold as 96-hour boxes for the shipment of sensitive samples, failed to performed as publicized by the manufacturers and did not keep temperatures between 2°C and 10°C for the duration of the tested 96-hour profiles. All three containers utilize vacuum-insulated panels as the shielding layer, but differ in the cooling method employed to maintain sample temperature. The Credo Cube and ThermoSafe containers make use of phase-change materials (panels or gel-packs) preconditioned to 4°C to prevent sample warming. This can be seen as relatively smooth and steady temperature increases in Figure 1A and C. In contrast, NanoCool uses an active evaporative cooling system, which can be observed as a flat line and stable temperature in Figure 1B. The active cooling effect can also be seen as small increases and decreases (peaks and dips) in sample compartment temperature in Figure 3.

5.2 Independent Qualification Data

The active cooling system performed nearly twice as well as the phase-change materials (55 hours for NanoCool under the ISTA-7D profile versus 31 and 28 hours for the Credo Cube and ThermoSafe). However, these data did not match results from an independent laboratory qualification provided by NanoCool (Figure 4). Independent testing of the 96-hour NanoCool container reported sample temperature maintenance of approximately 96 hours under the ISCgold profile and 108 hours under the ISTA-7D profile [8]. These results far exceed what was observed during equivalent experiments carried out in this proposal, as compared to our findings of 38 hours with ISCgold and 55 hours with ISTA-7D. Additionally, high variability in chilling performance was noticed during testing of the NanoCool box, perhaps due to manufacturing defects/standards of the active cooling lids. Unfortunately, NanoCool LLC was not amenable to

troubleshooting this difference in observed performance when approached for advice and assistance.



(A) ISCgold

(B) ISTA-7D

Figure 4. Independent laboratory qualification report on NanoCool long haul shipping systems.

5.3 Homogenization

Immediate homogenization of food samples was examined as a method of quickly reducing sample temperatures. The product chosen, the IKA Turrax tube-drive system, was capable of macerating room temperature (~25°C) raw ground beef in 60 seconds in pre-chilled buffer, immediately reducing specimen temperature to 4°C. This is in contrast to hand-stomached or non-stomached food samples that took 10 or more minutes to reach 10°C. However, we were unable to find a probe thermometer with sufficient speed and accuracy to record results, preventing satisfactory quantification of the results. Future experiments with higher precision thermometers will address this and evaluate its utility.

5.4 Temperature Logging

NFC LOG-IC temperature loggers from American Thermal Instruments (ATI) were utilized to track sample compartment temperature, as they provided the best "complete" system. This system included the reusable logger, a handheld reader, and software to track, log, and export data. A key manufacturer-advertised feature of the ATI logger was the ability to detect and download data from outside the package prior to opening through NFC or radio frequency. However, the data loggers failed to perform as marketed and we were unable to detect and read the loggers' data until it was removed from the insulated container and was held in close proximity to the handheld reader. Similarly, ATI's software was not user friendly and did not support easy programming of the logger or easy export of data. ATI no longer supports or sells the LOG-IC NFC logger, the handheld reader, or the LOG-IC software.

5.5 Cost

The NanoCool long haul shipper is $105 for the reusable insulated container and a single-use cooling lid; replacement lids are $29. The Credo Cube (Series 4 2896) costs $520 for the complete system (box plus phase-change gel panels). The ThermoSafe VIP box is $104, plus approximately $20 for sufficient phase-change gel-packs. With the exception of the NanoCool replacement lid, all components are reusable.

6.0 CONCLUSIONS

Basic sample processing at the point of collection was evaluated as a method of rapidly reducing sample temperature to the required range (4°C to 10°C) by utilizing the IKA Turrax container, which has plastic rotor elements capable of homogenizing samples in a pre-chilled buffer prior to packing and shipping. There are numerous homogenization protocols available on IKA's website, many for processing matrices more difficult than food (mammalian organ tissue, plant roots/seeds, wood, etc.), making the IKA Turrax system an appropriate tool for the rapid homogenization of food samples. Additionally, IKA offers container lids with a pierceable membrane, which eliminates the need to open samples when preparing food specimens for laboratory analysis. This is intended to not only reduce bacterial growth in suspect food, but also minimize technician handling (sample transfer and stomaching) of potentially hazardous samples. Qualitatively, we observed much more rapid sample cooling with the IKA Turrax tubes as compared to whole sample alone, whole sample plus pre-chilled buffer, or hand-stomached sample plus cold buffer. However, issues with available temperature sensing equipment obscured differences between the tested methods. Future experiments are planned with appropriate equipment to determine the possible benefits of point of collection sample homogenization.

Three commercially available off-the-shelf shipping systems, marketed for 96-hour transport, were evaluated for their temperature holding capacity under two industry-developed extreme summer shipping profiles. None of the boxes tested were capable of maintaining samples in the target temperature range (average external temperature >30°C) for the full 96-hour incubation. The best performing system, the NanoCool long haul shipper, kept samples under 10°C for 55 hours when exposed to an average external temperature of 30.4°C, but only 38 hours with an average external temperature of 31.8°C. Further insulating the box significantly extended its performance under extreme conditions by more than 40%, suggesting that simple modifications may enable this system to reach the 4-day or 96-hour goal. Additionally, at an external temperature of 25°C, the NanoCool system was able to keep the sample compartment temperature under 10°C for 102 hours, further indicating that performance is acceptable under milder environmental conditions.

Although the NanoCool shipper fell short of the 96-hour performance goal, it is inexpensive, approximately half the size of similar 96-hour shippers, and is easily "re-iced" due to a replaceable chilling unit in the lid for transit times longer than 55 hours. While only evaluated for the transportation of food samples in the present work, the NanoCool system would also be an appropriate method of shipping any temperature-sensitive sample within the continental United States or for regional shipping.

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LIST OF ABBREVIATIONS AND ACRONYMS

ATCC	American Type Culture Collection
ATI	American Thermal Instruments
CFU	colony forming unit
ISTA	International Safe Transit Association
NFC	near field capable
PBS	phosphate buffered saline
PCR	polymerase chain reaction
qRT-PCR	quantitative real-time polymerase chain reaction
TB	Terrific broth